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HUMAN WOUND REPAIR

II. Inflammatory Cells, Epithelial-Mesenchymal Interrelations, and Fibrogenesis

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ABSTRACT

Connective tissue repair was studied in a series of skin wounds in young adult males. The tissues were examined at 3, 12, and 24 hr, and at 2, 3, 5, 7, 14, and 21 days after wounding. The neutrophilic leukocytes contain within membrane-bounded vacuoles some fibrin and serum protein from the wound; however, most of the granulocytes lyse and release their cytoplasmic contents into the extracellular space. The mononuclear cells undergo a series of morphologic alterations during which they develop a modest amount of relatively poorly developed rough endoplasmic reticulum and an extensive system of smooth-surfaced membranes prior to active phagocytosis. They could be clearly distinguished from immature fibroblasts by the differences in the development of their organelles, particularly the rough endoplasmic reticulum. The perivascular connective tissue adjacent to the wound contains cells which appear like poorly developed or immature fibroblasts. The development of these cells into mature fibroblasts can be followed during the different stages of wound repair. Intimate contact was observed between basal cells of the regenerated epidermis and monocytes in the wound below: cytoplasmic projections of the basal cells extended beneath the basement lamina to the surface of the monocytes. Such contacts were seen only on the 4th–7th day after wounding. Their possible significance is discussed.

INTRODUCTION

Numerous ultrastructural studies of wound repair in experimental animals have provided interesting and useful information concerning the inflammatory response and connective tissue formation. Most of these investigations were performed with the guinea pig (1–7), the rat (8–12), and the rabbit (13). To date, little information has been available concerning these processes as they occur in man (14). This paper and the preceding one (15) comprise the first of a series of investigations into various aspects of human wound repair. These studies demonstrate the similarities in the inflammatory response and fibrogenesis between human wounds and wounds in experimenta animals, and present some new observations concerning relationships between epithelial cells and mesenchymal cells.

MATERIALS AND METHODS

The material consisted of the same series of human skin wounds described in the preceding paper (15). These wounds represent repair of linear skin incisions in the forearms of a series of young adult males. The wounds were removed serially at 3, 12, and 24 hr, and at 2, 3, 5, 7, 14, and 21 days after wounding, and were prepared as previously described

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(15). The tissue blocks were oriented so that each thin section contained a cross-section of each wound. Observations were made in an AEI EM6B, an RCA EMU3G, or a Siemens Elmiskop II electron microscope.

OBSERVATIONS

The Inflammatory Response

THE GRANULOCYTES: At 3 and 12 hr after wounding, the predominant cells in the wound are granulocytes, primarily neutrophilic leukocytes. These cells together with a few mononuclear cells can be seen by 3 hr. They are surrounded by serum protein and strands of fibrin that form a mesh-work throughout the wound. Similar observations were made at 24 hr (Fig. 1).

Distal to the wound margin, numerous neutrophilic leukocytes were observed in the process of emigrating from small vessels after 24 hr. After emigrating through the vessel walls, they could sometimes be seen adjacent to perivascular connective tissue cells that resemble poorly differentiated fibroblasts (Fig. 2), to be described below.

As the neutrophilic leukocytes move into the wound, many of them appear to undergo lysis and to release their cytoplasmic granules into the extracellular environment. These extracellular neutrophilic granules often are surrounded by a clear halo (Fig. 1). Some neutrophils can be seen to contain small amounts of fibrin and serum protein in membrane-bounded vacuoles.

By 72 hr, the neutrophils have decreased in number within the wound and many of them appear to have been ingested since they are present within large phagocytic vacuoles in macrophages that reached the wound during that time.

THE MONONUCLEAR CELL: A few mononuclear cells are present as early as 24 hr after wounding, and they increase in number on the 2nd and 3rd days, becoming the dominant cellular constituent of the wound by the 5th day.

Examination of the wounds at various time periods after wounding indicated that the mononuclear cells undergo a series of morphologic changes that accompany the development of phagocytic activity. These changes are described below.

After 24 hr relatively poorly developed monocytes are located in the perivascular connective tissue adjacent to the wound (Fig. 3). In contrast to the fibroblasts, which will be described subsequently, the monocytes characteristically have poorly developed rough endoplasmic reticulum and well-developed smooth endoplasmic reticulum and Golgi complex. The mitochondria have a relatively dense matrix and regularly arranged cristae. These cells also contain free ribosomes, a large, prominent, irregularly shaped nucleus, and an irregular, ruffled surface. Little evidence of phagocytic activity is seen in these cells at this early time.

In 2- and 3-day wounds, the monocytes contain an increased amount of rough endoplasmic reticulum, although this organelle remains poorly developed in contrast to that of the fibroblast. The cisternae of the rough endoplasmic reticulum have relatively few attached ribosomes, but many free ribosomes are found within the cytoplasm. Numerous mitochondria and relatively well-developed smooth endoplasmic reticulum and Golgi complex are present. A monocyte at this phase of development is shown in Fig. 4.

As noted in experimental wound healing in animals (18), the monocyte can be differentiated from the fibroblast by a relative paucity of rough endoplasmic reticulum. In addition, this reticulum contains relatively sparse aggregates of small numbers of attached ribosomes. In sharp contrast, the rough endoplasmic reticulum of the fibroblast is highly developed. This disparity in the development of rough endoplasmic reticulum, together with the differences in the appearances of mitochondria and smooth endoplasmic reticulum, permit one to distinguish between the monocyte and the fibroblast when seen together at the fine structure level (Fig. 5). Figs. 4 and 11 illustrate and stress these differences between the mature monocyte and the mature fibroblast.

Mononuclear cells that have migrated into the wound contain numerous membrane-bounded bodies within which can be seen material similar in density to the serum protein and fibrin present in the extracellular space (Fig. 6). In Fig. 6 the poorly developed rough endoplasmic reticulum of the monocyte is also apparent. Deposits of particulate material appearing like glycogen are often present within their cytoplasm.

At days 3 and 5, the monocytes contain increasingly larger amounts of extracellular material from the wound. Many large membrane-bounded structures containing deposits of varying appearances can be seen within these cells. Often these deposits appear as whorls of membranes that

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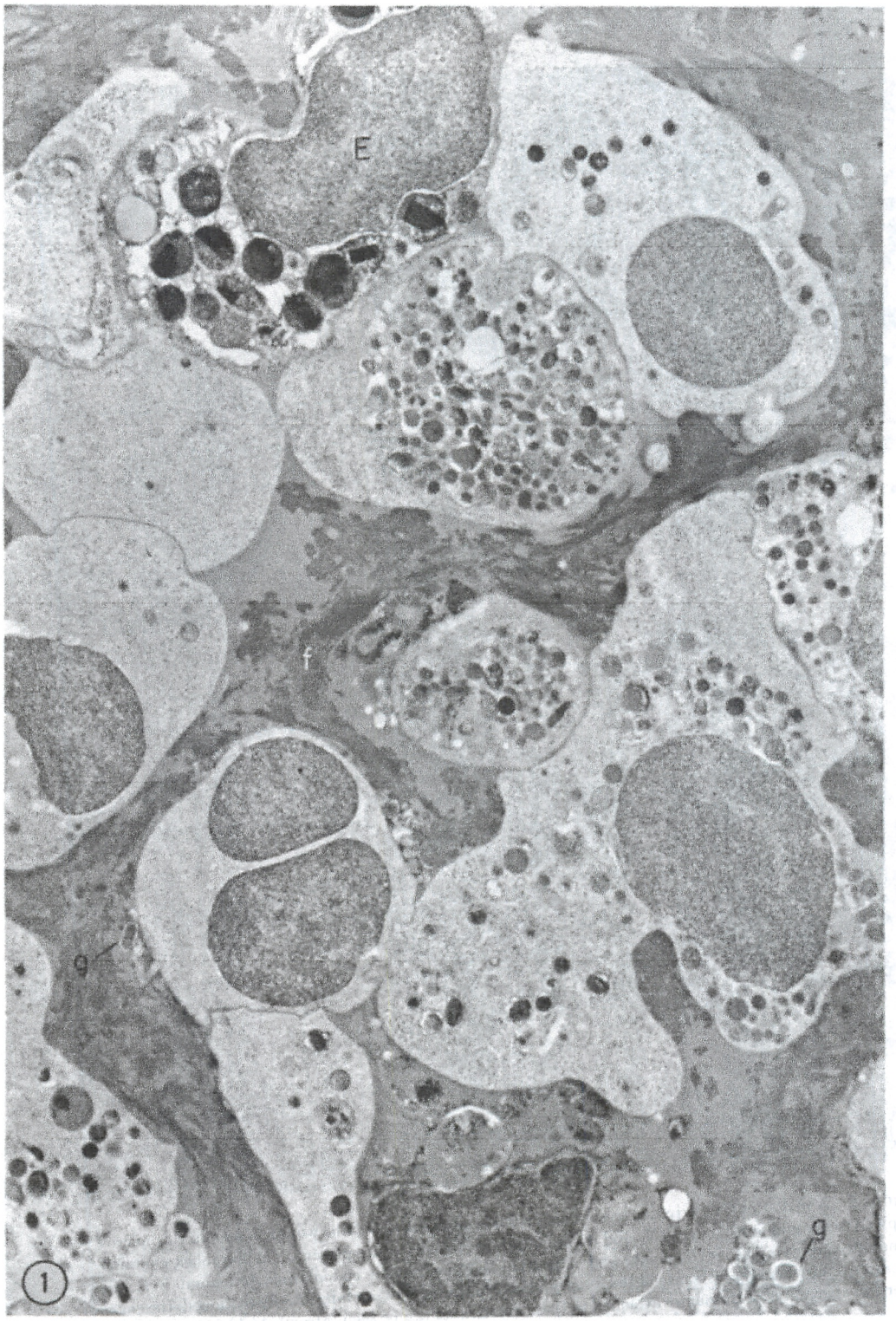

FIGURE 1 This micrograph displays a typical region from the center of a 24 hr wound. The cells appear to be largely granulocytes, most of which are neutrophils with an occasional eosinophil (*E*). Strands of fibrin (*f*) ramify throughout the extracellular space which appears dense because of its large content of serum protein. Free neutrophil granules (*g*), often surrounded by a somewhat clear halo, are seen within the extracellular space. × 11,000.

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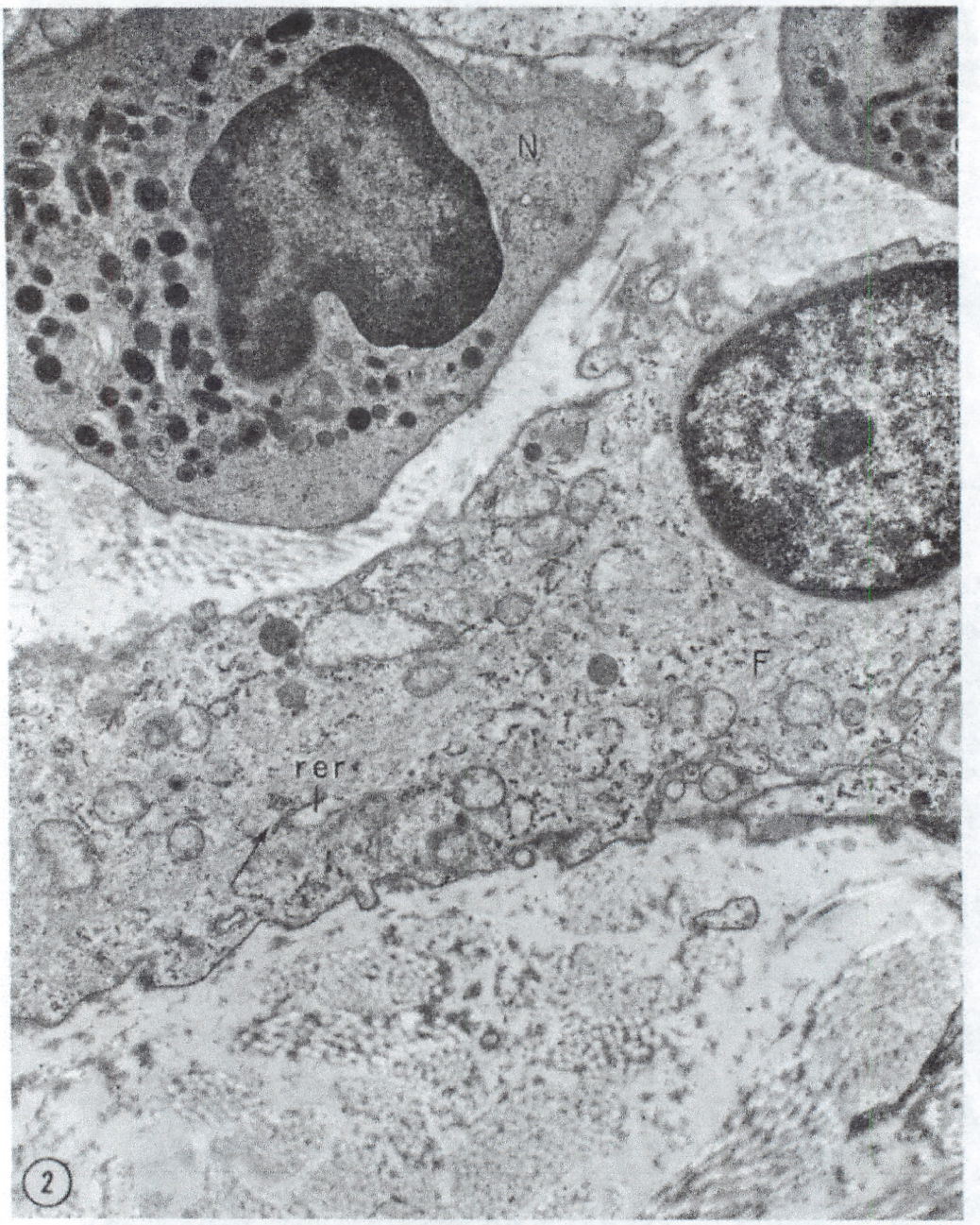

FIGURE 2 A region of the perivascular connective tissue adjacent to a 24 hr wound is seen in this micrograph. A neutrophilic leukocyte (*N*) and an immature fibroblast (*F*) are present. The fibroblast contains numerous fine cytoplasmic filaments and a poorly developed rough endoplasmic reticulum (*rer*). In several regions where the cisternae of the rough endoplasmic reticulum are tangentially sectioned, the characteristic aggregates of ribosomes attached to these membranes can be seen (arrow). × 12,000.

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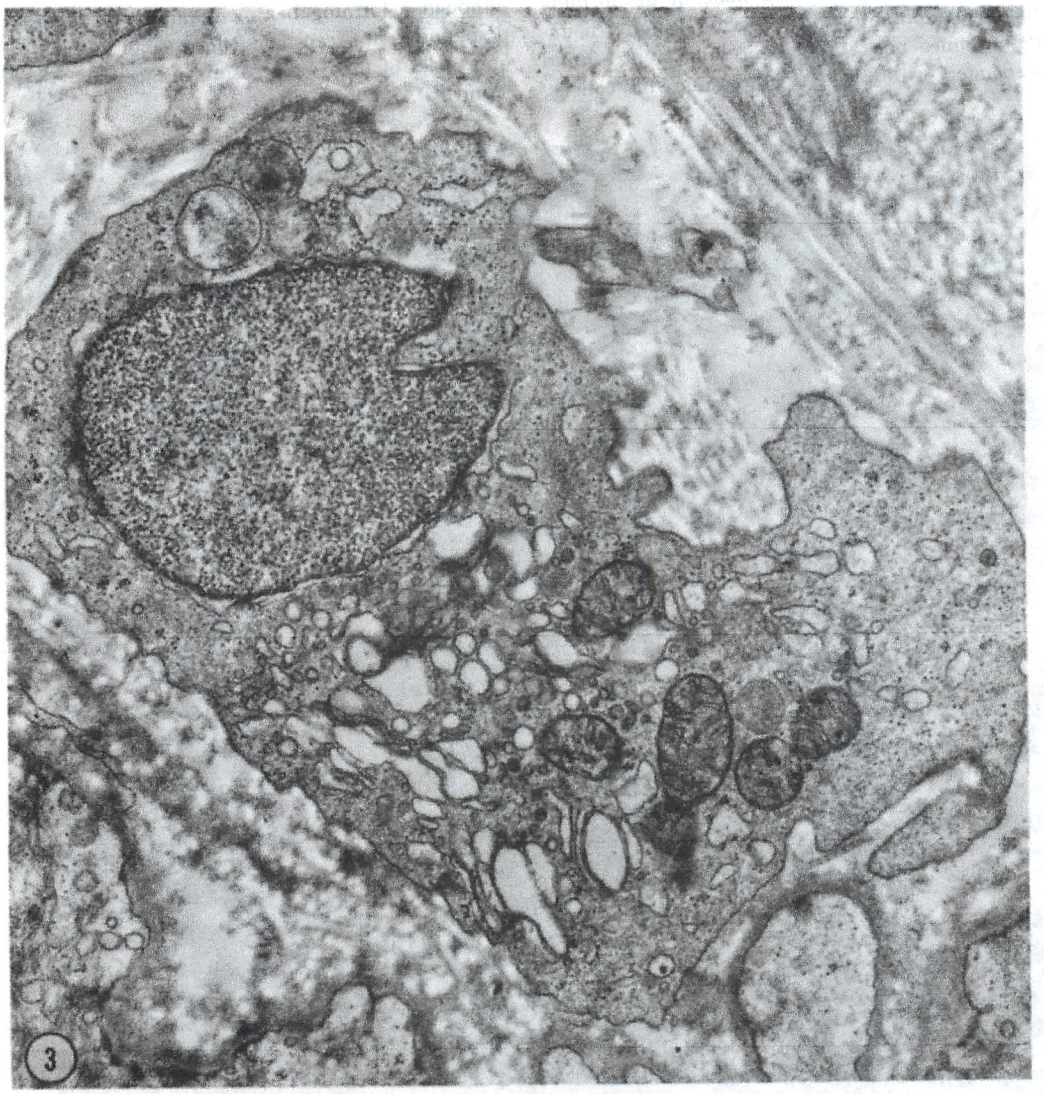

FIGURE 3 This micrograph shows a monocyte in the dermis adjacent to a 2 day old wound. This cell contains a well-developed Golgi complex, numerous profiles of smooth-surfaced membranes, and a poorly developed rough endoplasmic reticulum. The mitochondria have a relatively dense matrix and a somewhat regular arrangement of their cristae. × 19,000.

present a myelin-like structure or, in other cases, as bodies of varying sizes and densities (Fig. 7). The extracellular content of fibrin and serum protein diminishes during the same time interval. At the time when monocytes show phagocytic activity, relatively young, immature fibroblasts and some collagen formation can be seen in the wound (Figs. 5 and 8).

Interrelationships Between Mononuclear Cells and Regenerating Epidermis

An interesting feature frequently encountered in regions of the regenerating epidermis is the intimate contact which is established between cytoplasmic extensions of the basal cells of the epidermis and monocytes present within the wound (Figs. 9 and 10). In these regions, the

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basal cells send projections beneath the forming basement lamina of the epidermis. Neither hemidesmosomes nor a basement lamina are found associated with these cytoplasmic projections, although a forming basement lamina and hemidesmosomes are seen along the base of the cell at both sides of these cytoplasmic projections (Figs. 9 and 10).

FIGURE 4 The monocyte seen in this micrograph of a 3 day old wound contains an increase in the development of both its smooth-surfaced and rough-surfaced membranes. This particular cell has not yet engaged in phagocytosis. × 10,500.

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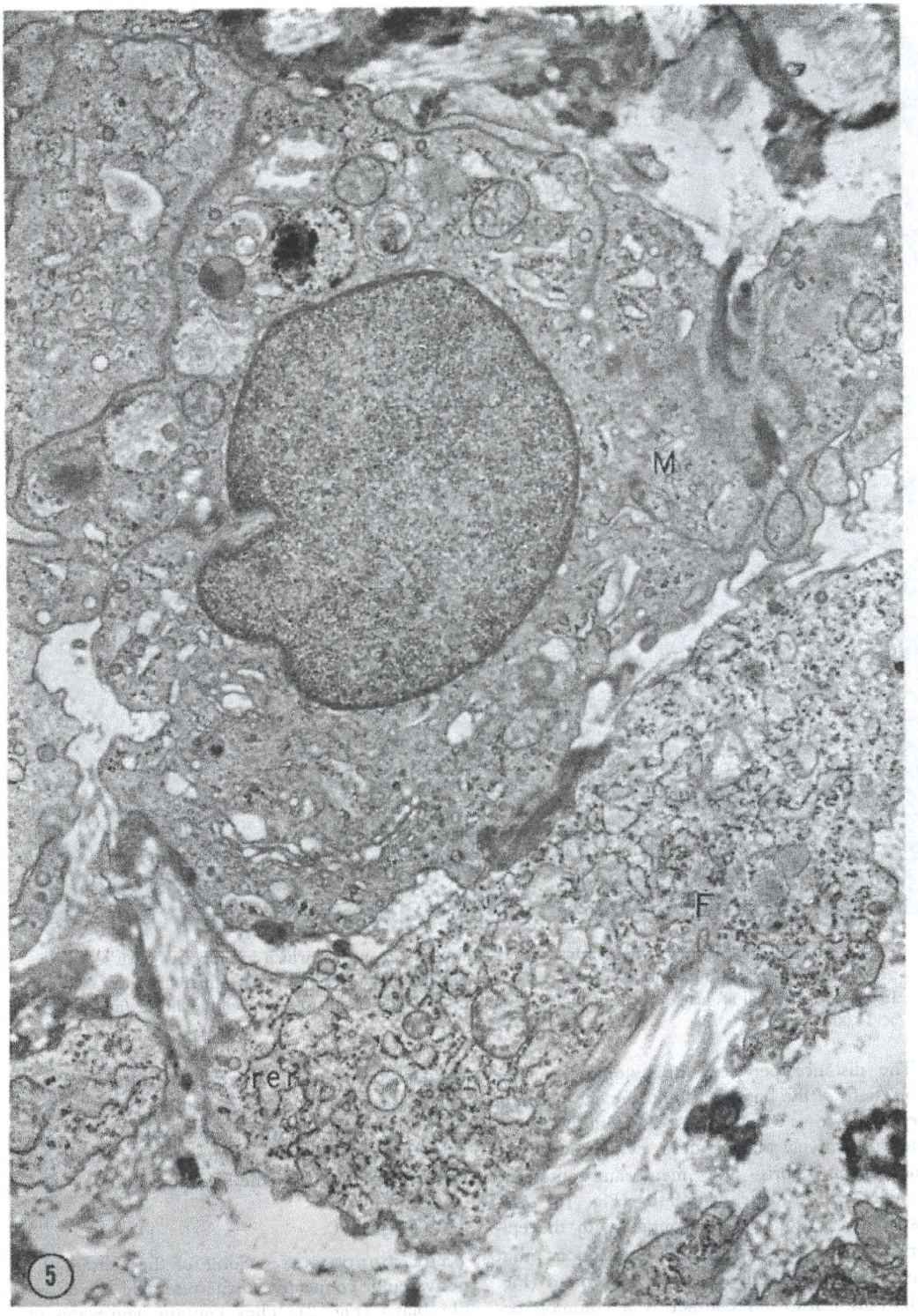

FIGURE 5 This micrograph is representative of the dermis immediately adjacent to a 3 day old wound. A relatively immature appearing fibroblast (*F*) and a monocyte (*M*) which has engaged in some phagocytosis can be seen. The difference in the morphology of these two cells is evident. The principal feature of the monocyte is the well-developed system of smooth-surfaced membranes, in contrast to the relatively greater amount of rough endoplasmic reticulum (*rer*) present in the fibroblast. × 11,500.

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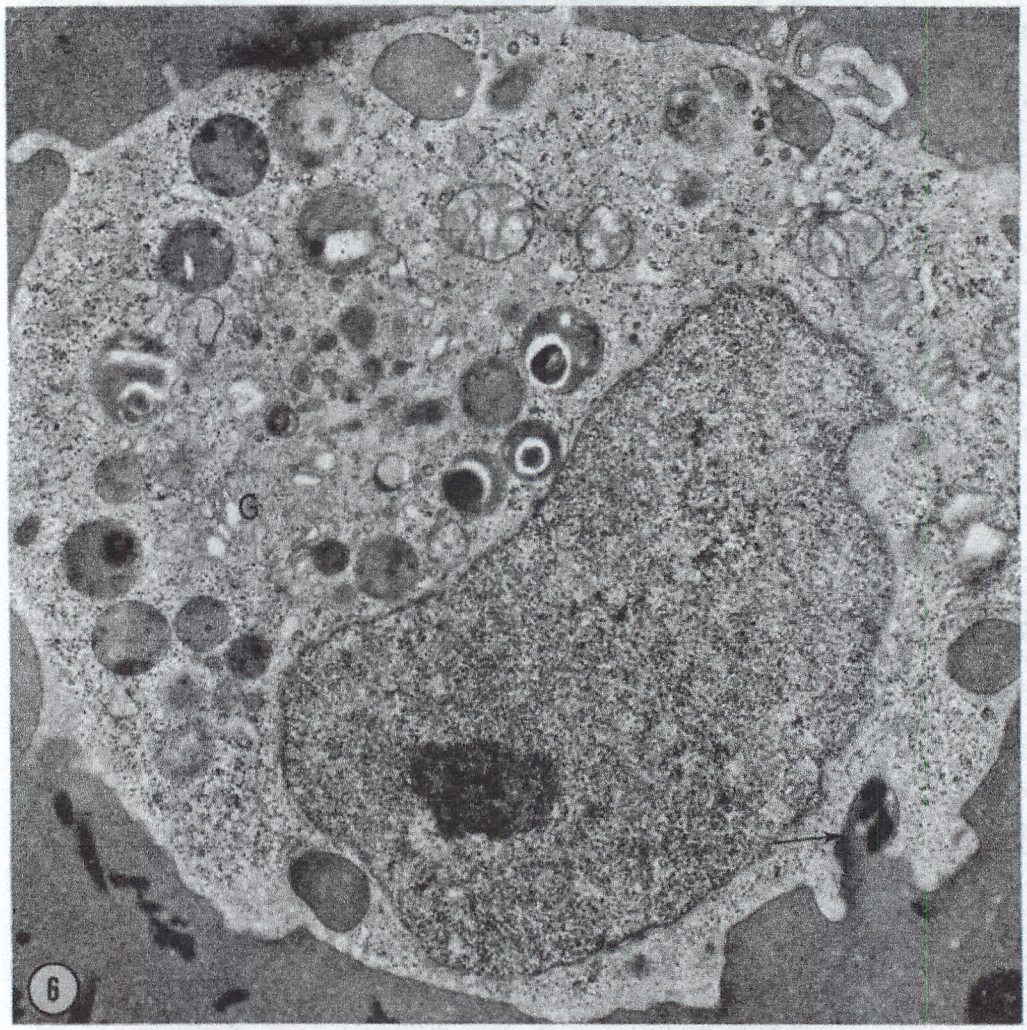

FIGURE 6 This micrograph demonstrates in a 24 hr wound a monocyte which has ingested both serum protein and fibrin within membrane-bounded bodies. The Golgi complex (*G*) is also apparent in this cell. In one site the cell seems to have partially surrounded a small amount of extracellular fibrin (arrow). × 14,000.

The distance between the cytoplasm of the projections of the basal cells and the cytoplasm of the monocytes is within 200 A. Some of the monocytes are actively phagocytic as evidenced by the amount of debris and membrane-containing vacuoles within their cytoplasm (Fig. 10). Occasionally, a monocyte in contact with the epidermis also has cytoplasmic projections that contact a second cell deeper in the wound (Fig. 10). This cell-to-cell contact is a common occurrence in the wounds at 5 and 7 days. Similar contacts between basal cells and fibroblasts are not apparent. At the time of these observations, relatively little collagen has formed and the epidermis rests on a dense mat of fibrin from which it is separated only by the basement lamina

Fibrogenesis

THE FIBROBLAST: Some of the cells in the perivascular connective tissue resemble immature fibroblasts (Fig. 2). They contain aggregates of large numbers of ribosomes attached to the mem-

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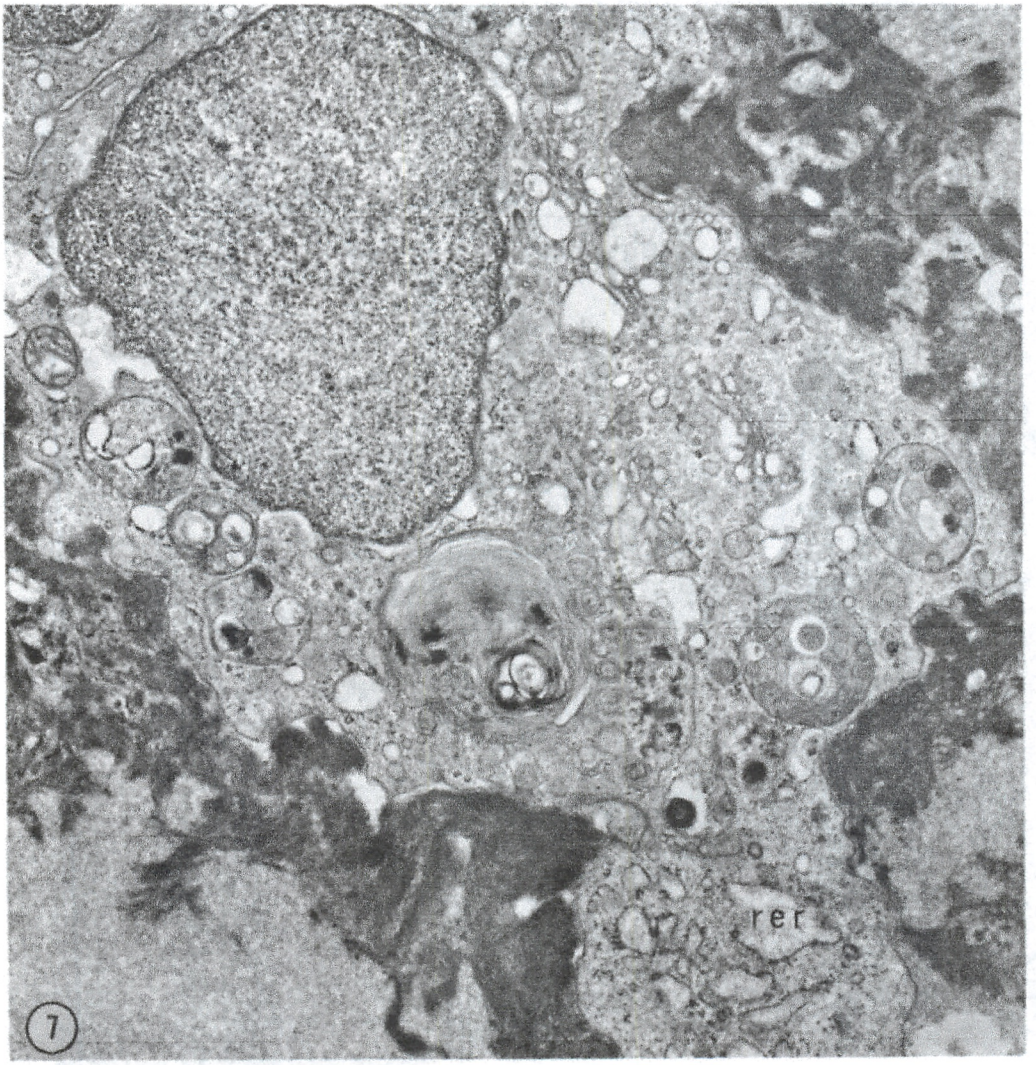

FIGURE 7 This micrograph shows a region in the center of a 3 day wound in which the monocyte has been actively engaged in phagocytosis. The cell contains numerous membrane-bounded bodies containing whorls of membranes and small deposits of varying densities. The rich development of smooth-surfaced membranes and the Golgi complex are evident in this cell, in contrast with the poor development of the rough endoplasmic reticulum (*rer*). The monocyte is surrounded by serum protein and strands of fibrin. × 14,000.

branes of the rough endoplasmic reticulum and large mitochondria with pale matrices and short cristae. Their rough endoplasmic reticulum is relatively poorly developed at first, and numerous fine filaments are dispersed throughout their cytoplasm.

After 7 days these cells have become mature fibroblasts and contain a more extensively developed rough endoplasmic reticulum (Fig. 11). The differences between the mature fibroblast and the immature fibroblasts shown in Figs. 2, 5, and 8 are evident. Although the more mature fibroblast also contains numerous fine filaments, large pale mitochondria, and a well-developed Golgi

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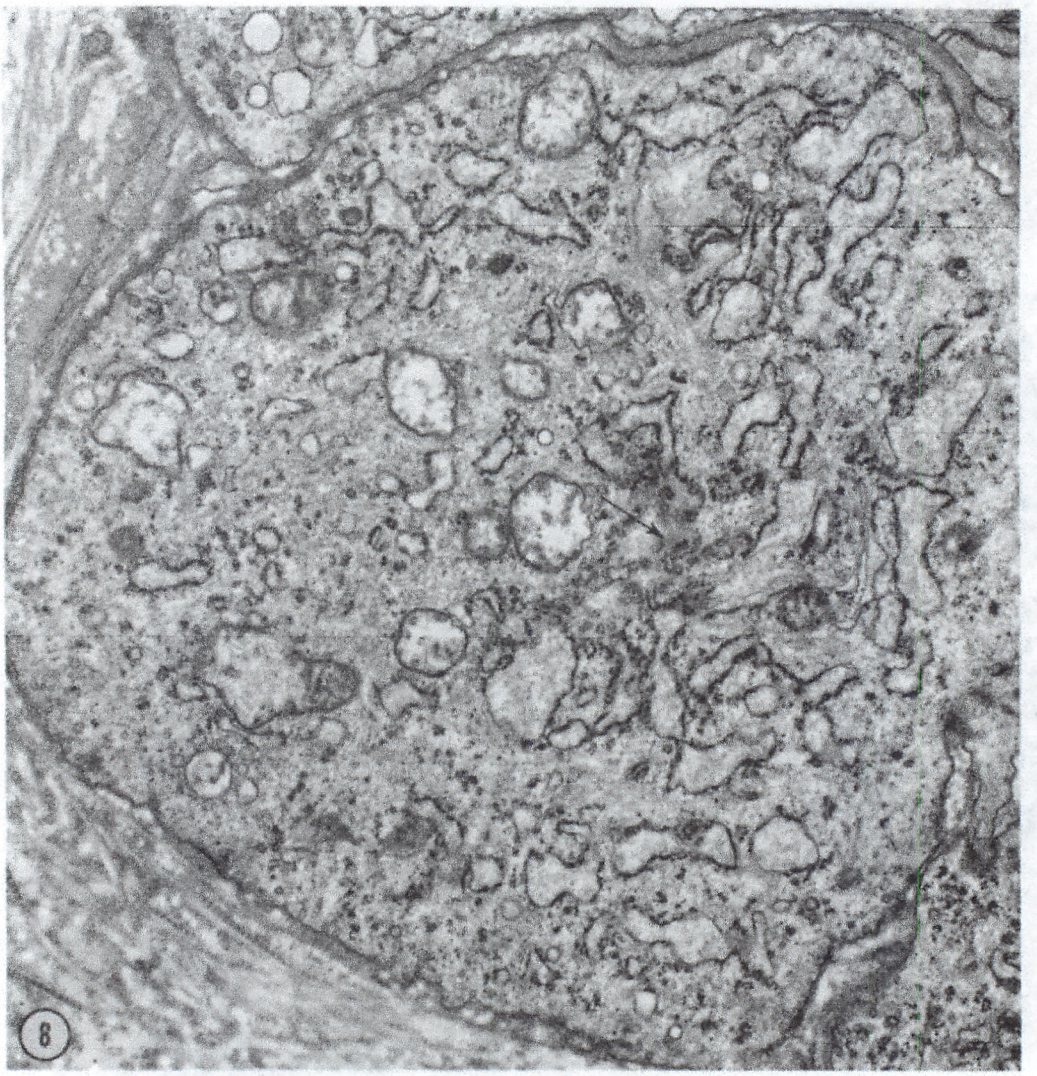

FIGURE 8 This micrograph of a region near the margin of a 3 day wound demonstrates a developing fibroblast. The increase in the amount of cisternae of rough endoplasmic reticulum with their characteristic attached aggregates of large numbers of ribosomes (arrow) can be seen. The mitochondria appear dilated and have a relatively pale matrix. Numerous fine filaments can also be seen within the cytoplasm. Many vesicles and small caveolae are present at the surface of this cell, together with small extracellular collagen fibrils. This cell appears to be intermediate in its development between the fibroblasts seen in Figs. 2, 5, and 11. × 14,000.

complex, all of which are similar in appearance to those seen in the less well differentiated cell, the mature fibroblast is rich in rough endoplasmic reticulum whose cisternae ramify throughout the cytoplasm and whose membranes contain aggregates of large numbers of attached ribosomes. Where the cisternal membranes are tangentially sectioned, the aggregates of attached ribosomes can be seen to consist of at least 20–30 ribosomes, often arranged in double rows or spirals similar to those described in experimental wounds in animals (Figs. 8 and 11) (5, 18).

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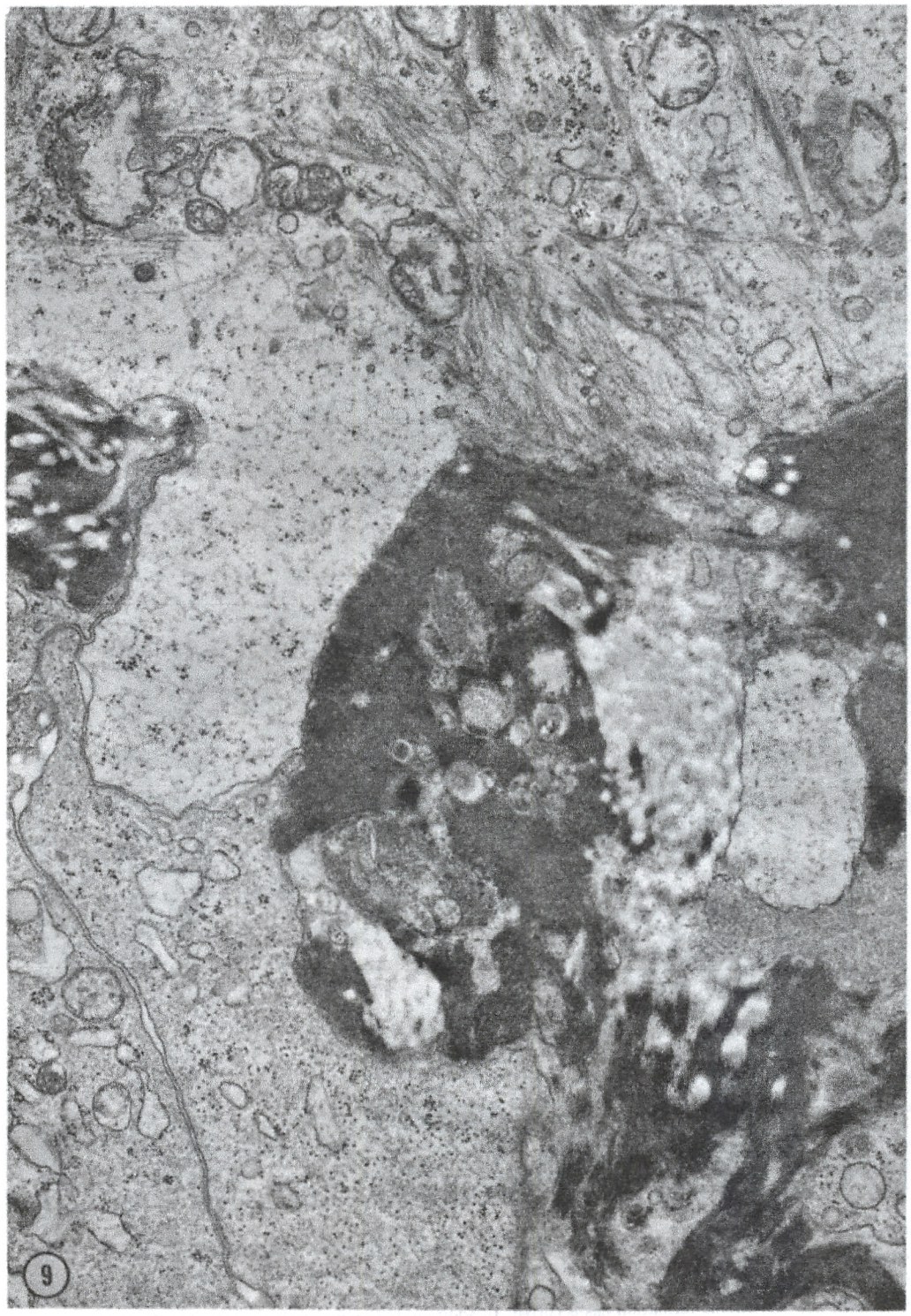

FIGURE 9 This micrograph shows the dermal-epidermal region of a 3 day wound. A cytoplasmic projection extending from a basal cell into the fibrin meshwork of the underlying wound can be seen. Neither hemidesmosomes nor a basement lamina are associated with this cellular projection. In a region immediately adjacent to this site, hemidesmosome formation and a basement lamina are apparent (arrow). The epidermal cell process is closely associated with the underlying mononuclear cell, and is characteristically pale with few organelles. × 17,000.

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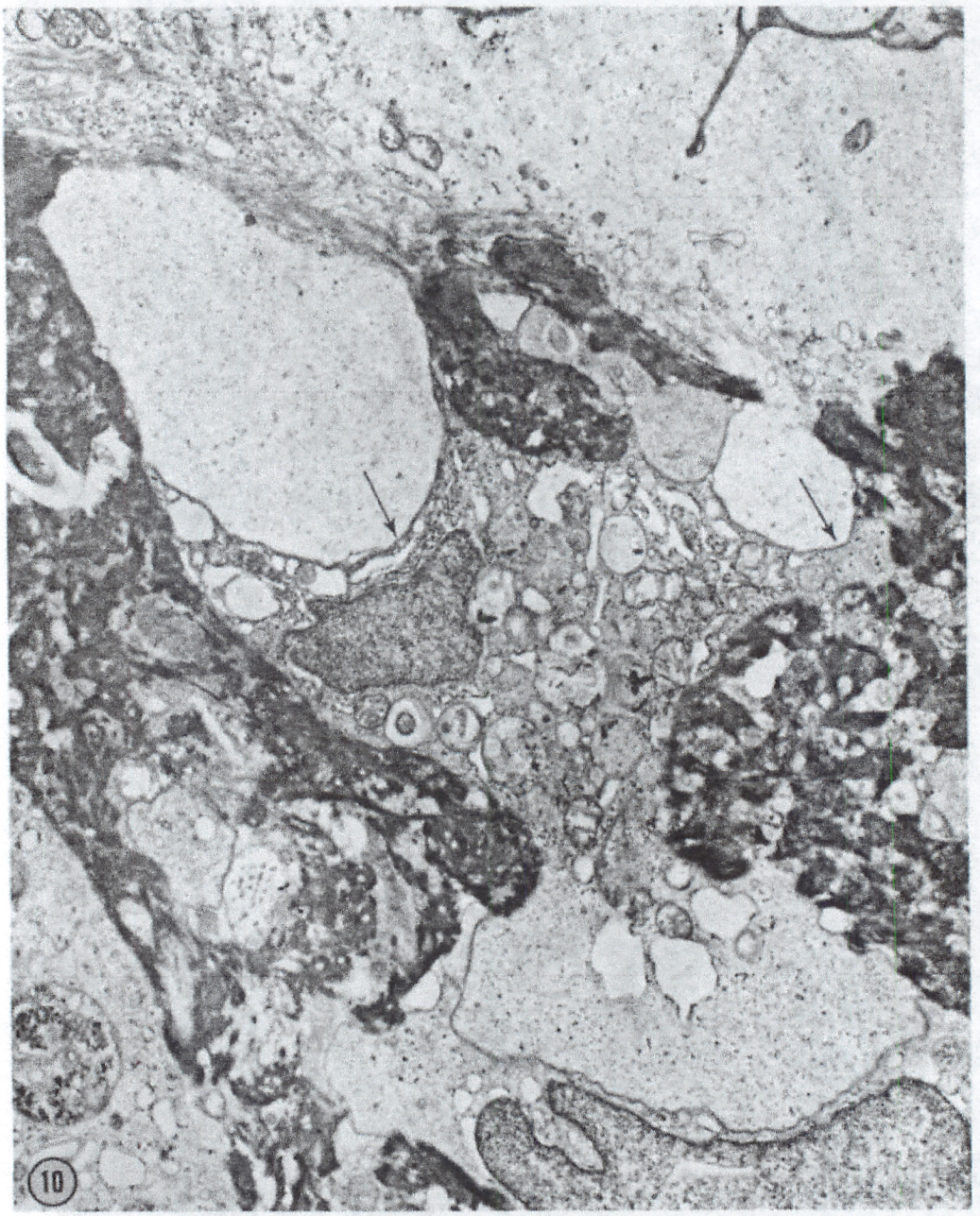

FIGURE 10 This micrograph is representative of a 3 day wound in which two cytoplasmic projections of a basal epidermal cell are intimately associated with that of an underlying mononuclear phagocyte (arrows). Each cytoplasmic projection of the basal cell is separated from the other by regions in which hemidesmosome and basement lamina formation has occurred. In addition, the monocyte has a cytoplasmic projection which is in intimate contact with a second mononuclear cell deeper within the wound. × 10,500.

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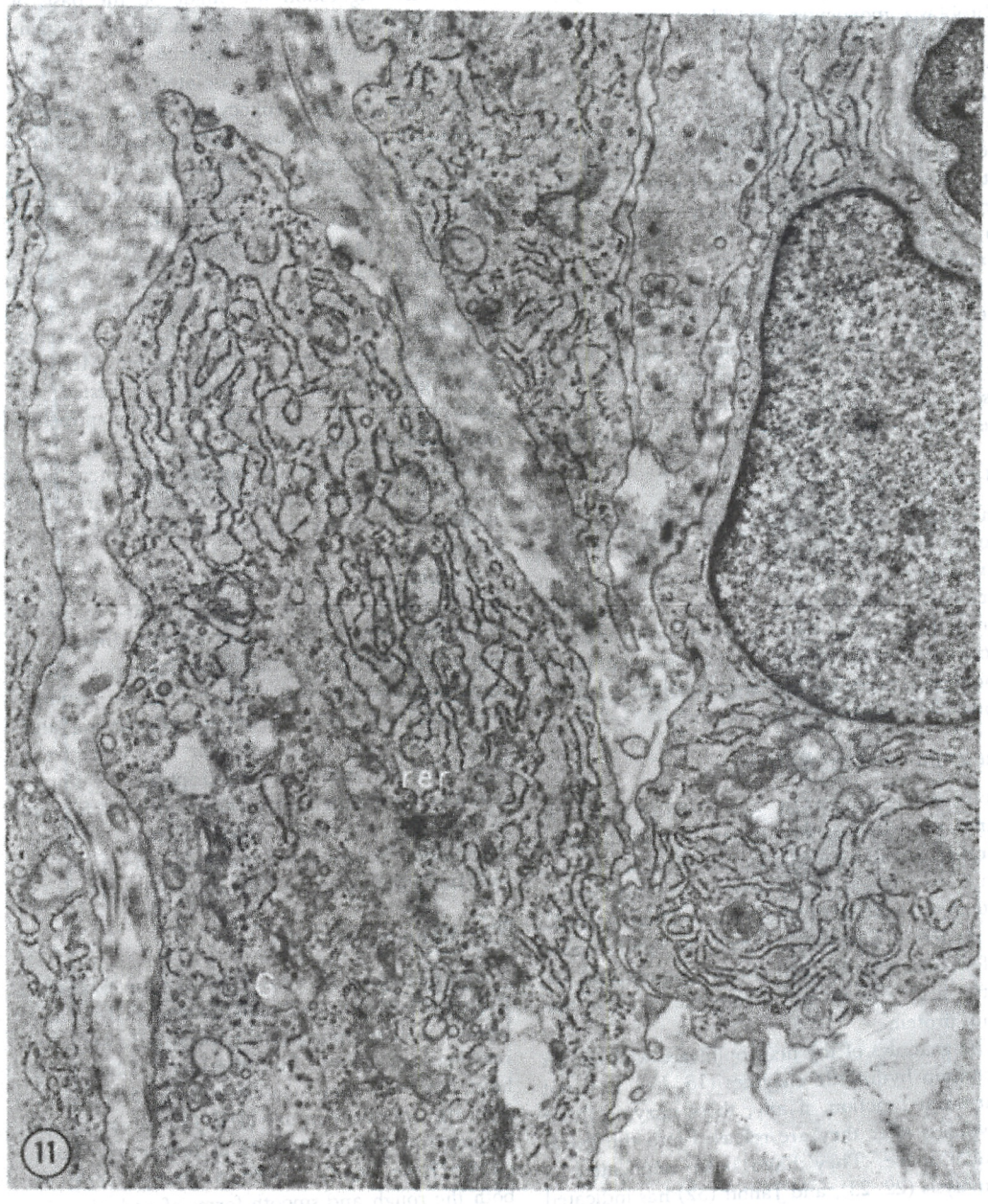

FIGURE 11 This micrograph illustrates a 14 day wound in which portions of several mature fibroblasts can be seen. At this stage the characteristic appearance of the fibroblast is evident. The highly developed rough endoplasmic reticulum (*rer*) and Golgi complex (*G*) together with numerous large, pale mitochondria are apparent in this cell. The fibroblasts are surrounded by numerous collagen fibrils of varying dimensions, sectioned both longitudinally and transversely. × 11,000.

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By the 14th day the wound consists largely of fibroblasts, monocytes, and blood vessels together with many collagen fibrils. At this stage the cisternae of the rough endoplasmic reticulum are clearly the dominant feature of the fibroblast. In numerous regions the cisternal membranes come close to the surface of the cell, and in many other sites small vesicles and caveolae often lie close to regions of rough endoplasmic reticulum cisternae in which the membranes lack ribosomes (Figs. 8 and 11).

Many small nonbanded fibrils (50–80 A) are often present within the extracellular space close to the surface of the fibroblasts. Collagen fibrils with a periodicity of approximately 700 A can also be seen. As the age of the wounds increases, the diameter of the collagen fibrils also increases.

DISCUSSION

The inflammatory response and fibrogenesis in human skin wounds are strikingly like those in skin wounds of experimental animals, no important differences being observed. The phenomenon of contact between epidermal cell and connective tissue cell has not been studied in the wounds in experimental animals, but is an obvious feature of the human wounds.

The Inflammatory Response

THE NEUTROPHILIC LEUKOCYTE: As in guinea pig wounds, the primary role of the polymorphonuclear neutrophilic leukocyte in human skin wounds does not appear to be phagocytosis. Instead, these cells become lysed and their granules are dispersed into the extracellular environment. A few neutrophils demonstrate phagocytic activity, largely involving the uptake of fibrin and serum protein.

The granules of the neutrophil are well known to contain hydrolytic enzymes (16, 17). It is not clear what function, if any, these enzymes have in wounds after they are released into the extracellular space. They have been implicated in fibrinolysis (22, 23), and Janoff (32) has indicated that proteolytic enzymes from human neutrophil granules can function at neutral pH. Cochrane and Aikin (31) have demonstrated that neutrophil enzymes can degrade vascular basement membrane, a collagen-like protein, although the pH in these conditions was not known. They felt that the pH would be lowered in regions where large numbers of neutrophils are concentrated in vivo. This suggestion was made on the basis of their observation that the pH in in vitro suspensions of neutrophils was lowered. In addition, Lazarus et al. (37) have isolated a collagenase from neutrophilic leukocytes, which possibly explains in part the observations of Cochrane and Aikin. This collagenase might serve also to degrade and subsequently help to remove the preexisting collagen remaining in the wound site.

Many extracellular neutrophilic granules surrounded by a clear halo can be seen in early wounds. The significance of this observation is not clear; it could be related to shrinkage artifact or to digestion of extracellular matter. Many of these granules are subsequently ingested by macrophages, as evidenced by their presence within phagocytic vacuoles.

Thus, our observations suggest that there may be two important functional roles for neutrophilic leukocytes in wound repair: cell lysis and phagocytosis.

As has been discussed before (18), there is experimental evidence to suggest that the acute inflammatory response may be an important requirement preceding orderly fibrogenesis. However, no direct experimental proof of this assumption has been yet forthcoming. Heppleston and Styles (19) have suggested that a product from appropriately stimulated macrophages is implicated in promoting fibrogenesis in silicotic fibrosis of the lung. A possible relationship between inflammation and fibrogenesis remains an interesting and important area for further investigation.

The Mononuclear Cells

Volkman and Gowans (20, 21) have clearly demonstrated that the mononuclear cells which behave as macrophages in various inflammatory reactions are derived from the blood. These cells have not been shown to be of local origin, i.e., from the site of the injury itself. Our observations demonstrate that monocytes at first entry into a wound have poorly developed complements of both the rough and smooth forms of endoplasmic reticulum, compared with active monocytes, and that within a relatively short period of time these monocytes ingest fibrin and serum protein. Riddle and Barnhart (22) and Barnhart (23) have demonstrated that the neutrophilic leukocyte has an important function in fibrinolysis. Our observations suggest that the monocytes play a significant role in the ingestion and digestion of these

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extracellular proteins. After continual ingestion of material from the extracellular space, these macrophages eventually become laden with numerous, large, membrane-bounded bodies containing membranous whorls of material together with unidentifiable debris. It is possible that formation of this lamellar-membranous material is one method by which the monocyte can efficiently store indigestible material.

During their development into active macrophages, the monocytes form an increased amount of both rough and smooth forms of endoplasmic reticulum. These organelles are possibly related to the formation of the hydrolytic enzymes which are used later by the macrophage to digest the ingested material. In the early phase of phagocytosis, the ingested material in the monocytes is in its native morphologic state. Subsequently, this phagocytosed native material is replaced by membranous and particulate components, possibly representing the residue of stored, indigestible matter.

The Source of the Fibroblasts

These observations provide no evidence to suggest any transformation of mononuclear cells to fibroblasts. Observations from a study by Ross and Lillywhite (24) indicated that those investigations purporting to demonstrate such a transformation (25–27) within either tissue culture or Millipore filter chambers were probably dealing with contamination with extraneous connective tissue cells removed together with the buffy coat that served as a source of the monocytes.

Our observations in human wounds further support the conclusions of Grillo (28) and Glücksmann (29) that wound fibroblasts are derived from perivascular connective tissue cells. During the earliest phase of wounding no obvious fibroblasts are present. Before recognizable fibroblasts enter the wound, cells that appear like immature fibroblasts are seen in the perivascular connective tissue (Fig. 2). These cells contain a large complement of cytoplasmic filaments dispersed throughout their cytoplasm. Their rough endoplasmic reticulum is relatively poorly developed although, in contrast to the situation in the monocyte, the aggregates of ribosomes attached to their rough endoplasmic reticulum membranes have the same pattern as that seen in mature fibroblasts. The mitochondria of these immature cells also appear similar to those seen in mature fibroblasts.

Thus, during the process of repair, the presumptive fibroblasts develop an extensive rough endoplasmic reticulum. By the time the wound is 14 days old they can be recognized as relatively mature fibroblasts containing all of the cytoplasmic features described earlier and are identical with fibroblasts in previously studied wounds in experimental animals (1–11, 18).

Epidermal-Connective Tissue Interaction

Cell contact regions between basal cells of regenerating epidermis and actively phagocytic mononuclear cells was a prominent finding in this study. In some sites it appeared as though there was a series of contacts between the epidermal cells and subjacent monocytes in the wound. In the regions of the epidermal projections, there were no associated hemidesmosomes nor a basement lamina.

Interaction between epithelial and connective tissues has been shown to be important in maintenance of the differentiated state of epithelial cells grown in vitro. Wessells (33), Grobstein and Cohen (34), and Dodson (35) have observed that, although direct contact with mesenchymal cells was not necessary to maintain epithelial differentiation, contact with a substrate containing an extracellular product of mesenchyme was important. In studies of thyroid epithelium Hilfer et al. (36) used fine structure as an additional criterion of cell differentiation. They observed that thyroid cells required the presence of homologous mesodermal cells in order to develop fine structural characteristics of mature thyroid epithelium. This raises the question of what role, if any, contact between epidermal cells and round cells of mesenchymal origin plays in wound repair.

In their studies of the formation of collagenase within wounds, Grillo and Gross (30) demonstrated that the main source of this enzyme appeared to be the epithelium. However, they did note that collagenolytic activity was enhanced when epithelium and connective tissue were placed together. It is not known whether this response resulted from contact between epidermal cells and connective tissue cells or between epidermal cells and collagen. It is not at all clear that our observations in human wounds are related

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to collagenolytic activity. The finding of epidermal cell-monocyte contact was so prominent during this phase of wounding that it remains an interesting and curious observation.

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